Stromal Staining for PINCH Is an Independent Prognostic Indicator in Colorectal Cancer[1]

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Abstract

Particularly interesting new cysteine−histidine−rich protein (PINCH), a LIM domain adapter protein that functions in the integrin and growth factor signal transduction pathway, is upregulated in stroma associated with many common cancers. The finding suggested that PINCH may be involved in promoting tumor−stromal interactions that support tumor progression, and, if so, tumors with abundant PINCH stromal staining may have a worse prognosis. To test this hypothesis, 174 primary colorectal adenocarcinomas with 39 distant normal mucosa samples and 26 metastases in the lymph nodes were studied by immunohistochemistry, and 7 additional colon tumors were studied by Western blot analysis and immunofluorescence. The abundance of PINCH protein in stroma increased from normal mucosa to primary tumor to metastasis ($P < .05$), and was more intense at the invasive margin than it was in the intratumoral stroma. Strong stromal immunostaining for PINCH was shown to predict a worse outcome (rate ratio 2.1, 95% CI 1.16−3.37, $P = .01$), independent of Dukes stage, growth pattern, and tumor differentiation. PINCH was detected in fibroblasts, myofibroblasts, and a proportion of endothelial cells of the tumor vasculature, supporting the involvement of PINCH in promoting tumor−stromal interactions that support tumor progression. Interestingly, stromal staining for PINCH was an independent prognostic indicator in colorectal cancer.

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Introduction

Particularly interesting new cysteine−histidine−rich protein (PINCH) was originally identified by Rearden [1] as a widely expressed, evolutionarily conserved protein that consists primarily of five LIM (double zinc finger) domains and contains an autoepitope homologous to ''senescent cell antigen.'' The *PINCH* gene is located on chromosome 2q12.2, and the protein functions as an adapter protein for signal transduction in the integrin and growth factor pathways [2−4]. Recently, PINCH protein was shown to be markedly upregulated in the tumor-associated stroma of many common cancers, including breast, prostate, lung, skin, and colon cancers [5]. In that study, PINCH was noted to be especially abundant in stromal cells at the invasive margin in these tumors, a region where signaling in the integrin and growth factor pathways is known to occur. The phenomenon of the ''intense at invasive edges'' was particularly observed in breast cancers ($n = 33$) and was not potentially described in colon cancers because only five cases were included.

Adapter proteins such as PINCH play important roles in the formation, compartmentalization, and stabilization of signaling complexes, and therefore increased PINCH abundance may augment signal transduction in stromal cells at the tumor edge, leading to downstream activation of pathways important in paracrine interactions with tumor cells. Because tumor−stromal interactions are important for cancer progression, it is possible that increased PINCH in stromal cells may have a role in promoting tumor progression, and, if so, tumors with abundant stromal staining for PINCH may be expected to have a worse prognosis. The aim of this study was to test this hypothesis by determining whether immunostaining of the tumor stroma for PINCH can predict outcome in colorectal cancer.

Materials and Methods

Patients

For immunohistochemistry, formalin-fixed paraffin-embedded tissue blocks were obtained from 174 randomly selected patients with primary colorectal adenocarcinoma who underwent

surgical resection at Linköping Hospital (Linköping, Sweden) and Vrinnevi Hospital (Norköping, Sweden). The study also included 39 normal mucosa specimens (29 of them were matched with primary tumors) taken from the margin of distant resection (distant normal mucosa) and 26 metastases (25 of them were matched with primary tumors) from the regional lymph nodes. Among the primary tumors, 96 cases had adjacent normal mucosa including dysplastic lesions. The patients' sex, age, tumor location, and Dukes stage were obtained from surgical and/or pathologic records at Linköping and Vrinnevi Hospitals. The mean age was 71 years (range from 34 to 94 years). The growth pattern was based on the patterns of growth and invasiveness. Differentiation was graded as better (good + moderate) and worse differentiation. Inflammatory infiltration was graded as weak and strong infiltration.

For Western blot analysis and immunofluorescence, seven additional colon cancer specimens were obtained freshly at the University of California, San Diego Medical Center, frozen immediately, and stored at −80°C until use.

Immunohistochemistry

The preparation, specificity, and reliability of the rabbit polyclonal PINCH antibody used in the study were as described previously [5,6]. Five-micrometer sections were deparaffinized and rehydrated, and then were treated by high-pressure cooking with 0.01 M Tris−EDTA buffer (pH 9.0) for 10 minutes and kept at room temperature (RT) for 30 minutes. The sections were incubated with 3% H_2O_2−methanol for 20 minutes and washed with phosphate-buffered saline (PBS; pH 7.4). The sections were further treated with protein block solution (Dako, Carpinteria, CA) for 10 minutes. After removing the solution, the sections were incubated with rabbit anti-PINCH at 2 μg/ml in antibody diluent (Dako) for 1 hour, followed by rinsing with PBS. Subsequently, the sections were incubated with a goat anti−rabbit/mouse, coupled with peroxidase provided by the Dako ChemMate EnVision Detection Kit (Dako) for 25 minutes, and washed with PBS. The peroxidase reaction, using 3,3′-diaminobenzidine tetrahydrochloride, was performed (Dako A/S, Glostrup, Denmark) for 8 minutes. Sections known to stain positively were included as positive controls. The negative control used PBS instead of the primary antibody. In all staining procedures, the positive controls showed clear staining, and there was no staining in the negative controls.

The sections were microscopically examined and scored independently by two of the authors without any information on the clinicopathologic data. PINCH staining was observed in the cytoplasm of fibroblasts in stroma. The staining intensity was scored as negative, weak, moderate, or strong, respectively, in 1) the entire tumor area, 2) tumor invasive margin, and 3) inner tumor area, irrespective of the percentage of positive cells. The percentage of stained cells was classified as < 25% staining, 25% to 49%, 50% to 75%, or > 75%, irrespective of the staining intensity. In the seven cases with discrepant scoring, a consensus score was reached by using a dual-headed microscope after reexamination and discussion. To avoid artificial effects, cells in areas with necrosis, with poor morphology, or in the margins of sections were not counted.

Western Blot Analysis

Frozen colon cancer tissue was thawed and mechanically dissociated using 1/4-in. stainless steel beads and a Mini-Beadbeater (Biospec Products, Bartlesville, OK) into the lysis buffer, 1% sodium dodecyl sulphate (SDS)/PBS containing a protease inhibitor cocktail (Complete; Roche, Indianapolis, IN). Protein concentrations of the lysates were determined by the DC protein assay (BioRad, Hercules, CA). Samples in loading buffer were boiled for 5 minutes in the presence of 2-mercaptoethanol and dithiothreitol. Solubilized proteins were separated by electrophoresis in 10% SDS polyacrylamide gels and transferred to nitrocellulose (Hybond-ECL; Amersham, Piscataway, NJ) by electroblotting in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. Equivalency of protein transfer was confirmed by staining the nitrocellulose membrane with Ponceau S. Nitrocellulose membranes were blocked for 30 minutes with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), pH 7.5, and then reacted overnight at 4°C with rabbit anti-PINCH at 1 μg/ml in 5% nonfat milk/TBS-T. Reactions were detected using horseradish peroxidase−conjugated anti−rabbit Ig (Amersham) at 1:5000 for 1 hour at RT followed by enhanced chemiluminescence (ECL; Amersham).

Immunofluorescence

Colon cancer frozen sections were air-dried overnight, fixed in cold acetone for 10 minutes, and blocked with 1% bovine serum albumin (BSA; Vector, Burlingame, CA) for 30 minutes. Sections were reacted with rabbit anti-PINCH at 10 μg/ml for 1 hour at RT, washed in TBS, and then reacted with Alexa-Fluor 488 goat anti−rabbit Ig (1:250; Molecular Probes, Eugene, OR) for 1 hour. Subsequently, sections were reacted with either mouse anti−human smooth muscle actin (SMA) (1:50; Dako) or with mouse anti−human CD31 (1:20; Dako) for 1 hour at RT, washed, and then reacted with Alexa-Fluor 546 goat anti−mouse Ig (1:250) for 1 hour. After washing, sections were mounted with Slow-Fade (Molecular Probes) and examined by fluorescence microscopy.

Statistical Analysis

The significance of the difference in intensity of PINCH expression between normal mucosa samples and primary tumors and metastases was tested by chi square analysis or McNemar's method. The relationships between PINCH expression and other factors were examined by chi square analysis. The relationship between PINCH expression and survival was tested using Cox's proportional hazard model. Survival curves were calculated using the Kaplan-Meier method. Two-sided *P* values of < 5% were considered as statistically significant.

Results

PINCH Expression in Normal Mucosa, Primary Tumor, and Metastasis

PINCH staining was present in the cytoplasm of fibroblasts in the stroma, whereas normal epithelial and tumor cells did not show any staining (Figure 1).

Table 1 presents staining intensity for PINCH in the distant normal mucosa, adjacent normal mucosa, entire tumor of primary tumors, invasive margin of primary tumors (excluding nine cases that did not have visible invasive margin), and metastases in the lymph nodes.

The intensity of PINCH expression was increased from distant or adjacent normal mucosa (no significant difference in the staining between distant and adjacent normal mucosa, $P = .74$) to primary tumor ($P = .0004$, $P < .0001$) to metastasis ($P = .003$) either in the unmatched or matched cases (matched distant or adjacent normal mucosa *versus* primary tumor, $P = .01$, $P < .0001$, and primary tumor *versus*
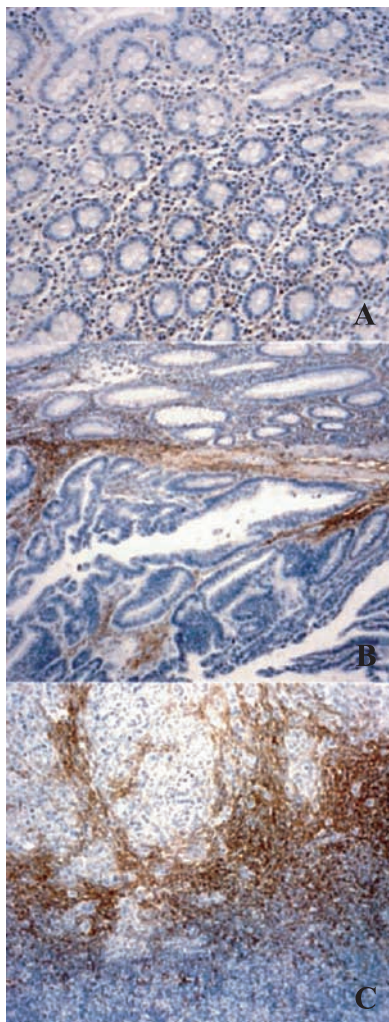

Figure 1. *A case presented PINCH immunohistochemical staining in the cytoplasm of fibroblasts, but not in the normal epithelia and tumor cells. (A) Weak staining in distant normal mucosa. (B) Increased expression in adjacent normal mucosa and even stronger staining in primary tumor. (C) The strongest expression in lymph node metastasis.*

metastasis, $P = .02$). Figure 2 presents the seven groups of PINCH staining patterns in 14 cases that had a complete data set including distant and adjacent normal mucosa, primary tumor, and metastasis. The staining intensity tended to be increased from distant to adjacent normal mucosa to primary tumor to metastasis (Figure 1, $A-C$). Six cases (groups 1, 3, and 7) showed increased staining from the distant normal mucosa to the primary tumor to metastasis; four cases (groups 2 and 5) showed increased staining from the distant normal mucosa to primary tumor, but were equal in primary tumor and metastasis. The remaining four cases (groups 4 and 6) showed the same staining in the distant normal mucosa and primary tumor but increased in metastasis. Regarding adjacent normal mucosa including dysplastic lesion, comparing with distant normal mucosa, 9 of 14 (groups 1, 2, 3, and 5) showed increased staining, two (group 4) showed the same, and three (groups 6 and 7) presented weaker staining. Comparing with primary tumor, 8 of 14 adjacent mucosa cases (groups 1, 2, 6, and 7) showed decreased staining and the remaining six (groups 3, 4, and 5) showed equal staining.

Comparing the PINCH intensity at the invasive margin with that in the inner tumor area, 112 (68%) had stronger staining at the invasive margin (Figure 3), 41 (25%) showed the same staining, and only 12 (7%) had weaker staining.

Considering staining percentage in 172 primary tumors (excluding two cases that had small stained areas), 23% showed < 25%, 23% showed 25% to 49%, 32% showed 50% to 75%, and 22% showed > 75% staining. We did not evaluate the staining percentage at tumor invasive margin, in normal mucosa, and in metastasis due to the small stained areas.

PINCH Expression in Primary Tumors in Relation to Clinicopathologic Variables

According to the similarities of the clinicopathologic features, the cases with negative, weak, and moderate stainings were grouped as a weakly staining group, and the cases with strong staining were grouped as a strongly staining group. Similarly, the staining percentage was classified as low expression and high expression using 50% as a cutoff point, regardless of the staining intensity.

As shown in Table 2, the frequency of strong PINCH expression was higher in tumors with better differentiation ($P = .02$) and weaker inflammatory infiltration ($P = .04$). Besides, we did not find associations of PINCH expression with other factors ($P > .05$).

Furthermore, patients with strong PINCH-stained tumors at the invasive margins had a poorer prognosis than those with weak staining ($P = .049$; Figure 4). Even in multivariate analysis, the expression was still related to survival, independent of sex, age, tumor location, Dukes stage, growth pattern, differentiation, and inflammatory infiltration ($P = .01$; Table 3).

Neither the intensity nor the percentage of PINCH expression in the entire primary tumor was significantly related to the clinicopathologic factors studied above ($P > .05$, data not shown).

Table 1. PINCH Staining Intensity in the Distant Normal Mucosa, Adjacent Normal Mucosa, Primary Tumor, and Metastasis.

Location	Number	Negative Staining (%)	Weak Staining (%)	Moderate Staining (%)	Strong Staining (%)
Distant normal mucosa	39	1 (2.6)	13 (33.3)	18 (46.1)	7 (18)
Adjacent normal mucosa	96	3 (3.1)	37 (38.6)	43 (44.8)	13 (13.5)
Primary tumor					
Entire tumor	174	1 (0.6)	26 (15)	60 (34.4)	87 (50)
Invasive margin	165	1 (0.6)	28 (17)	43 (26)	93 (56.4)
Metastasis	26	0	2 (8)	1 (4)	23 (88)

Western Blot Analysis

Lysates from seven fresh frozen colon cancer tissues contained differing amounts of PINCH protein (Figure 5, lanes 4–10), consistent with the variability in PINCH protein expression found by immunohistochemistry on the tissue sections. As previously described, the antibody was raised to a full-length recombinant human PINCH six-histidine fusion protein (rPINCH) [5,6]. As show in lanes 1 to 3, the reaction of PINCH antibody with the rPINCH fusion protein used as the immunogen results in the detection of multiple bands. PINCH protein migrates in polyacrylamide gel as a 37-kDa monomer (lane 1), a 75-kDa apparent dimer (lanes 1 and 2), and an anomalous migration band at about 50 kDa (lane 3), even with adequate reduction of the sample using 2-mercaptoethanol and dithiothreitol. The same multiple bands are seen when immunoblots of rPINCH are stained with anti-HIS to detect histidine residues, verifying that all the bands correspond to the recombinant protein and not to cross-reacting protein. Even other PINCH antibodies produced using other recombinant PINCH immunogens show the same pattern of multiple bands [3], indicating that authentic PINCH migrates in SDS gels at several molecular weights. These results are not likely to be caused by degradation as they are found in freshly isolated cell lysates prepared with protease inhibitors. Because the bands seen on our Western blots of human colon tissues (lanes 4–10) are the same as those found with rPINCH (lanes 1–3), it is our conclusion that these bands represent authentic PINCH and not cross-reacting protein. The antibody gives exceptionally clean staining showing no reaction in the absence of PINCH (e.g., there is no immunostaining of collagen in tissue sections).

Immunofluorescence

Immunofluorescence of colon cancer frozen sections showed PINCH immunostaining of the tumor-associated stroma (Figure 6), confirming the results found by immunohistochemistry of formalin-fixed, paraffin-embedded colorectal cancer tissue sections. Immunofluorescence also revealed the presence of endothelial cells in the tumor-associated stroma (staining for CD31; Figure 6A) and of myofibroblasts (staining for SMA; Figure 6B). Some, but not all, endothelial cells that stained for CD31 costained for PINCH (Figure 6A), indicating that a proportion of endothelial cells in the tumor vasculature expresses PINCH protein. Dual immunofluorescence showed colocalization of PINCH and SMA staining (Figure 6B), indicating that tumor-associated myofibroblasts express PINCH protein.

Discussion

Immunostaining for PINCH in cancers was investigated only by one study carried out on various common cancers, in which staining intensity was increased in tumor-associated stromal cells noted at the invasive margin [5], which led us to hypothesize the linkage of PINCH expression with tumor
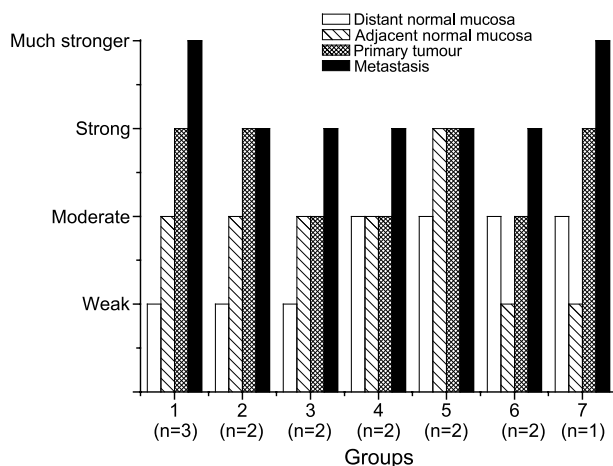

Figure 2. *The seven groups of PINCH expression patterns in 14 cases that had a complete data set including the distant normal mucosa, adjacent normal mucosa, primary tumor, and metastasis.*
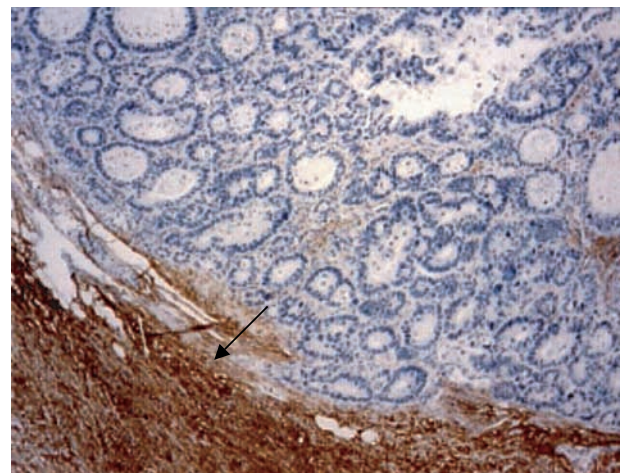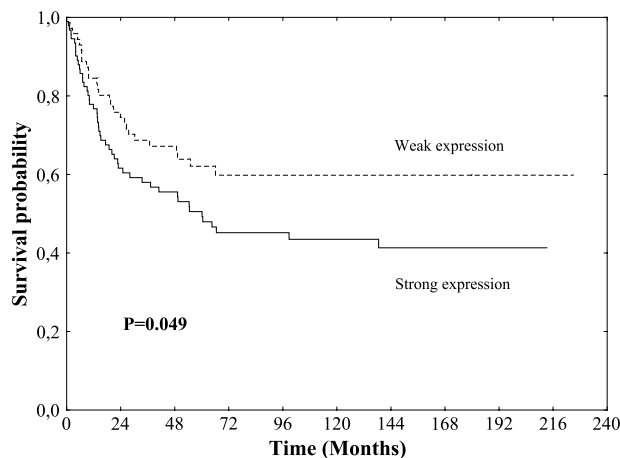

Figure 3. *Expression of PINCH protein at the invasive margin (arrow) was much stronger than in the inner tumor area.*

Table 2. The Relationship of PINCH Expression at the Invasive Margin of Primary Tumors with Clinicopathologic Variables.

Variable	PINCH Expression at Tumor Invasive Margin		
	Weak (%)	Strong (%)	P
Sex			
Male	38 (53)	53 (57)	.59
Female	34 (47)	40 (43)	
Age (year)			
≤ 70	31 (43)	40 (43)	1.00
> 70	41 (57)	53 (57)	
Tumor location			
Right colon	27 (38)	34 (37)	.91
Left colon	13 (18)	15 (16)	
Rectum	31 (43)	43 (47)	
Dukes stage			
A	10 (14)	8 (9)	.66
B	24 (33)	27 (30)	
C	25 (35)	33 (37)	
D	13 (18)	21 (24)	
Growth pattern			
Expansive	38 (54)	41 (46)	.30
Infiltration	32 (46)	48 (54)	
Differentiation			
Better	42 (58)	70 (76)	.02
Worse	30 (42)	21 (24)	
Inflammatory infiltration			
Weak	48 (80)	77 (92)	.04
High	12 (20)	7 (8)	

invasiveness. In this study, stromal staining for PINCH was shown, in general, to be minimal in stromal cells adjacent to areas of normal colonic epithelium and to be modestly to strongly positive in stromal cells adjacent to colon cancer cells. Stromal cells at the invasive edge typically had more intense staining for PINCH than those within the tumor, whereas intense stromal staining for PINCH was also noted in lymph node metastases. The pattern of stromal staining for PINCH in colorectal cancer confirms the pattern found in the prior study [5] in a much larger series of patients.

This study shows for the first time that stromal staining for PINCH is an independent prognostic indicator in colorectal cancer. Multivariate analysis showed that PINCH immunostaining predicted outcome independent of sex, age, tumor

Figure 4. PINCH protein expression at the invasive margins in relation to survival in patients with colorectal cancer.
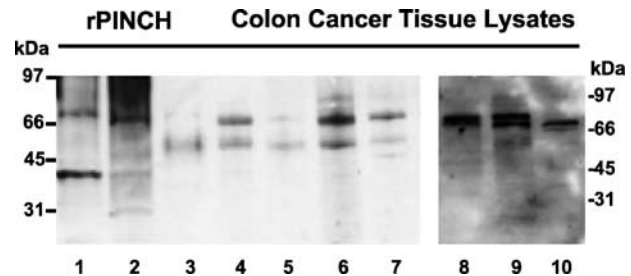
Figure 5. Western blotting. Lanes 1 to 3: Recombinant full-length six-histidine−PINCH fusion protein (rPINCH) as a monomer at about 37 kDa, apparent dimer at about 75 kDa, and anomalous migration band at about 50 kDa. Lanes 4 to 10: The lysates contained variable amounts of PINCH protein that migrated primarily as an apparent dimer.

location, Dukes stage, growth pattern, differentiation, and inflammatory infiltration. It is particularly notable that strong stromal immunostaining for PINCH at the invasive margin was associated both with better differentiation and worse survival. Although better differentiation is usually considered as a sign of favorable prognosis, the prognostic value of histologic grade is still controversial. The finding raises the possibility that it may identify a subset of patients with colorectal cancer who have aggressive disease in spite of favorable morphology.

Inflammatory infiltration is known to be a reflector of tumor-associated immune response and is generally considered as cytotoxic for the tumor cells. The prognosis advantage of strong inflammatory infiltration in colorectal tumors has been demonstrated [7,8]. Myofibroblasts have been considered to be associated with desmoplastic stromal responses to tumor. Myofibroblasts are proposed to

Table 3. Multivariate Analysis of PINCH Expression, Sex, Age, Site, Dukes Stage, Growth Pattern, Grade of Differentiation, and Inflammatory Infiltration in Relation to Survival in Colorectal Cancer.

| Variable	Number	Cancer Death Rate Ratio	95% CI	P
PINCH				
Weak	57	1.0	−	.01
Strong	79	2.1	1.16−3.37	
Sex				
Male	82	1.0	−	.67
Female	54	0.8	0.53−1.48	
Age (year)				
≤ 70	63	1.0	−	.85
> 70	73	0.9	0.57−1.58	
Tumor location				
Proximal	54	1.0	−	.18
Distal	82	0.7	0.41−1.18	
Dukes stage				
A + B	58	1.0	−	< .0001
C + D	78	4.0	2.19−7.37	
Growth pattern				
Expansive	61	1.0	−	.01
Infiltration	75	2.0	1.13−3.38	
Differentiation				
Better	98	1.0	−	.0003
Worse	38	2.8	1.62−4.85	
Inflammatory infiltration				
Weak	119	1.0	−	.89
Strong	17	1.1	0.40−2.86	
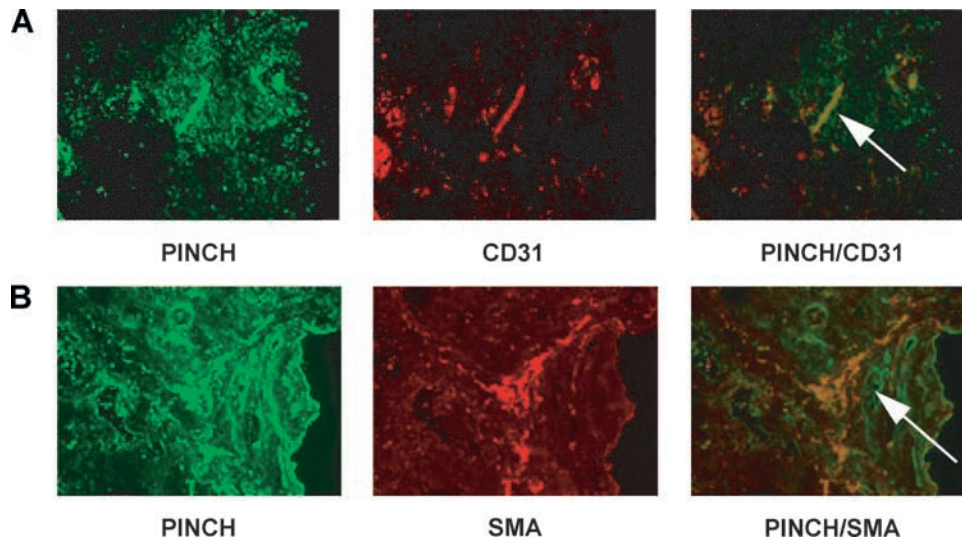
Figure 6. *Immunofluorescence staining for PINCH is green, for CD31 and SMA is red, and for costaining is yellow. (A) Endothelial cells (arrow) of blood vessels within the tumor-associated stroma costain for PINCH and CD31. (B) Region (arrow) of the stroma containing myofibroblasts costains for PINCH and SMA.*

form a barrier to the migration of immunocompetent cells toward the tumor, and hence to reduce immune surveillance. In colon cancer stroma, there is a negative correlation between inflammatory infiltration and the presence of myofibroblasts [9]. In this study, strong stromal immunostaining for PINCH was also associated with lack of inflammatory infiltration, and PINCH was shown by immunofluorescence to be present in stromal myofibroblasts, suggesting that the upregulation of PINCH in myofibroblasts may be the tumor-activated reaction against inflammatory cell infiltration, leading to tumor progression. PINCH was also shown to be present in a proportion of endothelial cells of the tumor vasculature, suggesting that PINCH protein is upregulated in tumor angiogenesis, which is particularly important and indispensable for tumor growth and metastasis.

Taken together, the finding reported here, that PINCH is upregulated in specific cells of the tumor-associated stroma, including fibroblasts, myofibroblasts, and endothelial cells, supports the hypothesis that PINCH is involved in promoting tumor–stromal interactions that support tumor progression. Interestingly, strong immunostaining stroma for PINCH at the invasive margin is an independent prognostic indicator in colorectal cancer.

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